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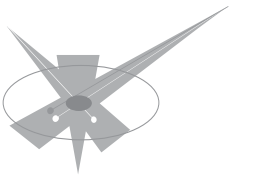

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Long-term changes of prostacyclin secretion in radiation--induced myelopathy

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Summary

Background We have previously reported the short-term changes in prostacyclin profile after irradiation of rat cervical cord.

Aim Present research investigated the long-term changes of prostacyclin content.

Materials/Methods Wistar rats in groups of five were irradiated with doses of 2, 4, 6, 15, 25, 30Gy and a single group of 25 with 35Gy X-rays. After 26 and 39 weeks, prostacyclin content was quantified by 6-keto-prostaglandin-F1α (prostacyclin stabilized metabolite). Specimens were stained routinely for histological studies.

Results The 50% latent period and effective dose were 14.86±1.16 weeks and 25.66±0.54Gy ($p<0.0001$), respectively. Average ratios of 6-keto-PG-F1α for doses of 2–30Gy were between 78.33–12.93% and 79.48–99.96% for 26 and 39 weeks, respectively. Prostacyclin level after 35Gy shows approximately a 7:1 ratio in comparison to the control group ($p<0.002$). Histopathological changes in glial and vascular tissues were diagnosed and scored. Prostacyclin bimodal profile was observed.

Conclusions Radiation can cause complex fluctuations of prostacyclin in association with marked histopathological changes.

Key words **spinal cord • radiation myelopathy • prostacyclin • histopathology • rat**

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Background

Radiation myelopathy is known as one of the most important complications of radiation in radiotherapy of patients. Destructive changes of the white matter and other nervous tissues are the major histopathological aspects. Dose response curves for treatment of malignancies distinctly show a strict margin between virtual 100% tumoricidal dose and neurotoxicity occurrence in spinal cord as a normal tissue [1]. It was shown that a single dose of 35Gy X-ray to the spinal cord can cause limb paralysis in rat models with a latency of 19.0±0.3 weeks [2].

Different types and methods of irradiation have been listed for different strains of animals, indicating dose dependency, latency, relative biological effectiveness (RBE), histopathology and pathogenesis of radiation myelopathy [3]. Recently we reported the short-term changes of prostacyclin secretory profile after various irradiation doses [4]. Histological observations showed that radiation with doses of a few Gy or more in excess of the tolerance level induces more or less uniform histological changes [1,5,6].

Aim

Based on vascular theory it is postulated that endothelial morphologic changes are associated with changes in prostaglandins, including prostacyclin, which plays a major role in haemodynamic alterations. Our short-term study showed fluctuations in the concentration of prostacyclin early after irradiation [4]. The authors studied late histopathology and enzymatic changes after doses of 2–35Gy.

Materials and Methods

Animals and irradiation

Male Wistar rats weighing 150–200g were fixed on a jig under gentle anaesthesia. The C1–T2 segment of the spine was irradiated through two parallel opposed ports with an orthovoltage machine (200kVp, 1.5mmCu HVL, focal skin distance 25cm) at a dose rate of 1.53Gy/min at the depth of the spinal cord. Doses of 2, 4, 6, 15, 25 and 30Gy were administered using a circular 3.0cm diameter localizator [7]. One group of animals (25 rats) was irradiated with a dose of 35Gy for evaluation of survival rate and to produce the clinical manifestation of radiation myelopathy. Five rats were included in each dose group in addition to the age-matched control group. The animals were sacrificed chronologically at five time points of 24 hours, 2, 13, 26 and 39 weeks post-irradiation. Rats irradiated with 35Gy were followed for symptoms of radiation myelopathy to get radiobiological data on latent symptoms and lethal dose.

Sample preparations

Following decapitation under ether anaesthesia, rats' neck regions were opened posteriorly and the spinal cord was removed after laminectomy. Then the spinal cord was cut rapidly and segmented into three pieces for enzyme immunoassay (EIA) and histopathological studies with transmission electron and light microscopes.

Measurement of spinal cord PGI2

Spinal cord samples were analyzed for prostaglandin I2 (PGI2 or prostacyclin) concentration by enzyme immunoassay kit (Cayman Chemical Co.). For this purpose, samples were homogenized with weight proportion of 0.1M perchloric acid and centrifuged at 4°C for 10 minutes at 3000rpm. Then 100–150µl supernatant was removed for dilution (1:5 and 1:10) and poured into a 96 well EIA kit. This kit measures 6-keto prostaglandin F1α (6-keto PGF1α), the main metabolic product of PGI2. The concentration of 6-keto PGF1α is directly proportional to PGI2. Data of 6-keto PGF1α concentrations were normalized in the form of the percent of the comparative control groups. Changes in PGI2 concentration were plotted against time and irradiation dose using Sigma-Plot version 8.

Histopathologic study

The samples were studied histopathologically for cellular and subcellular changes using electron (TEM) and light microscopes. Preparation of samples for the light microscope followed the routine procedure of fixation, embedding, cutting (4µm thick) and staining. TEM preparation consisted of fixation, embedding, semithin formation, griding/grinding and toluiden blue staining. Examination was done under appropriate magnification to observe morphological changes in endothelial cells.

Actuarial analysis was carried out on survival data. Quantification of histologic changes was scored based on one to three pluses in comparison to the age-matched control group. We used Student's t-test for comparison of histopathologic and PGI2 concentrations between irradiated and

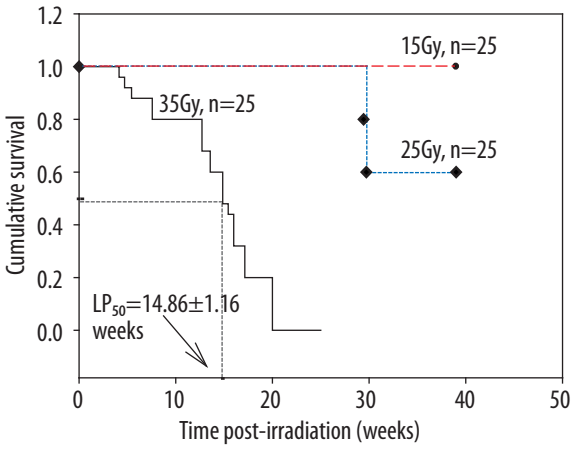


Figure 1. Kaplan-Meier analysis of Survival Function.

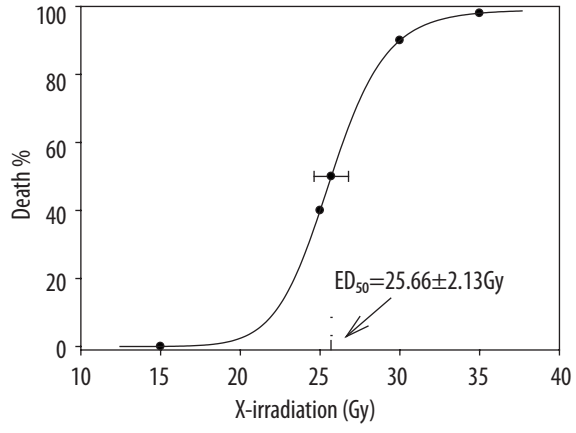
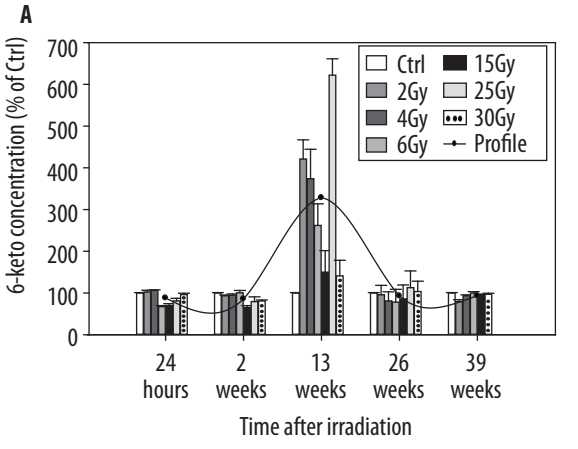


Figure 2. Dose-response curve for incidence of death due to radiation myelopathy (error bar shows mean ±SE and N=25).



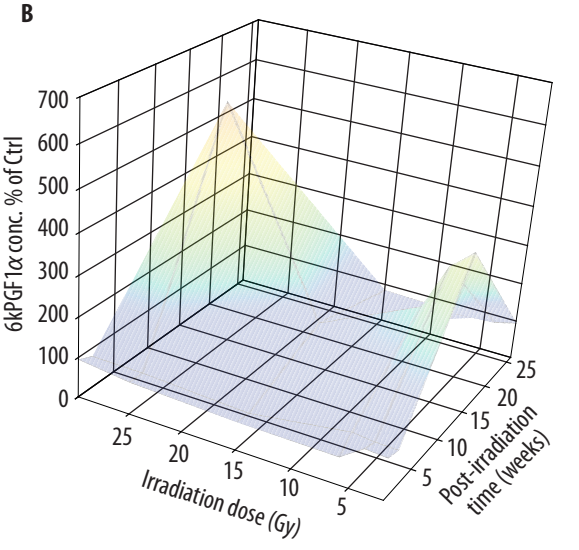


Figure 3. Profile of PGI2 changes in long-term study: (**A**) Detailed presentation of time-wise profile of prostacyclin changes (not scaled); this diagram shows concentration ratios as defined by PGI2 (irradiated)/PGI2 (age-matched control), and means ±SE are shown as a percentage at confidence limit of 95%. Dots indicate mean value of PGI2 at each time point. (**B**) 3D profile of prostacyclin changes as a function of time and dose.

control groups. Spearman's test was also used to evaluate the correlativity between PGI2 and histopathological changes.

Results

Survival data on the irradiated rats are shown in Figure 1. For the 35Gy group, the 50% latent period (LP_{50}) and mean latency period were 14.9±1.2 and 14.2±0.9 weeks (95% confidence limit 12.3–16.1 weeks), respectively. The obtained LP_{100} was 20 weeks post-irradiation. Dose response curve based on these findings is plotted in Figure 2. Effective dose of 50% (ED_{50}), which is the radiation dose that produces radiation myelopathy in 50% of rats, was found to be 25.7±2Gy within the 95% confidence interval of 24.6 and 26.7Gy.

Prostacyclin changes

Prostacyclin level in animals irradiated with 35Gy at the time of induction of paralysis and just prior to death showed a steep increase in comparison to corresponding control rats (approx. 7:1 with $p<0.002$, not shown). After 26 weeks of irradiation, overall prostacyclin concentrations changed little with radiation dose. In contrast to a significant decrease in PGI2 levels at 4 and 6Gy, a significant increase was observed at 25 and 30Gy. Details of PGI2 changes after different time points are shown in Figure 3A. Initially a positive part of this

Gaussian curve is seen at dose of 2Gy (p=0.07), decreasing at dose of 4Gy (p=0.01). At dose of 6Gy it shows a concentration not very different from control (p=0.007). In the high dose region there is an increase after 25 and 30Gy (p=0.004 and 0.05, respectively). After 39 weeks of irradiation, prostacyclin concentration decreased significantly in the low dose region of 2, 4 and 6Gy from 78.4% to 93.3% of control (p=0.001, 0.002 and 0.04, respectively). In the high dose region the decrease was significant after 15Gy (p=0.004) but it was not significant in the 25 and 30Gy irradiated groups (Figure 3A). Overall changes in PGI2 concentration in different time and irradiation dose are shown in Figure 3B. A double-peak action of prostacyclin response to irradiation was seen after 13 weeks in both low and high dose regions, which may indicate bi-modal parameters contributing to radiation myelopathy.

Histopathological changes

Different kinds of pathologic evidence were observed which are well established as signs of radiation-induced myelopathy. They included severe demyelination, white and grey matter atrophy, necrosis accompanied by atrophy, gliosis, and vasculopathy consisting of vascular rupture and haemorrhage. The incidence of histopathological lesions was both dose- and time-dependent. Substructural changes were observed even at a dose lower than 6Gy. However, we observed rats with clinical manifestation of radiation myelopathy after 25Gy. They showed paralysis of limbs and severe urine discontinuity and were sacrificed just prior to death after 26–28 weeks post-irradiation. In these animals necrotic and atrophic changes in spinal cord specimens were observed. The most apparent pattern after 26 weeks was an increased density of vessels mimicking revascularization at the irradiated volume (Figure 4A).

Findings after 39 weeks were similar to the findings after 26 weeks but more intense. Severe multiple necrotic areas were seen in sections obtained from irradiated rat cervical spinal cord after 25Gy. Fibrin deposition and replaced collagen fibres as fibrosis were seen in the interstitial spaces (Figure 4A).

Animals irradiated with a dose of 35Gy were examined histologically just prior to sacrifice for symptoms of radiation myelopathy. Marked pathological changes both in vascular and white matter parenchyma consisting of vascular rupture, wall thickening, severe necrosis, demyelination and glial proliferation were observed and are shown in Figure 4C.

Histopathological changes revealed with both light microscope and TEM are listed in Table 1. Changes encountered with TEM are at the level of the vascular endothelial cells. Photomicrographs were obtained at different times post-irradiation with different doses (Figure 5). They show changes at the subcellular level including nuclear membrane, cytoplasmic and basal cell membranes, and also alterations in endothelial cell organelles.

Discussion

Animals exposed to single doses ≤15Gy (i.e. 2, 4, 6 and 15Gy) did not reveal any specific clinical manifestation of radiation myelopathy. After doses >15Gy the probability and also the severity of radiation myelopathy increases. Our results are similar to the results of other reported experimental studies which have used different types of radiation [8–11]. The latency observed in our study is about 4 weeks shorter than the report of Hopewell et al. 1987 [12]. A similar discrepancy was also reported by Okeda et al. 1998 [10]. It may have been caused by the greater volume of irradiated spinal cord which may decrease the latency [13,14].

Similar results of histopathology and radiobiology were shown in the study on the effects of a carbon ion beam on the thoracic spinal cord by Shinobu and Okeda 2001 [6]. The ED_{50} for hind-limb paralysis derived from the dose-response curve was 18.5Gy and the RBE was 1.38. The equivalent dose of the X-ray beam was 25.5Gy and they found that 67% of animals at 20Gy and 100% of animals at 25 and 30Gy showed hind-limb paralysis, which is identical to our results, but at 16–20 weeks after irradiation [10].

Substantial data suggest that the prostanoids are particularly important in inhibiting smooth muscle cell proliferation, preserving tissue viability and organ function in experimental models of ischaemia. Ischaemic tissues are subjected to decreased oxygen tension (hypoxia), decreased blood flow, accumulation of metabolites, and parenchymal cell damage. The changes of prostacyclin against various damaging agents were studied *in vitro* and *in vivo*. There are two target cell populations involved in radiation myelopathy: I) vascular endothelial cells and II) glial cells (especially oligodendrocytes) and prostacyclin may originate from both cell types [3,15,16].

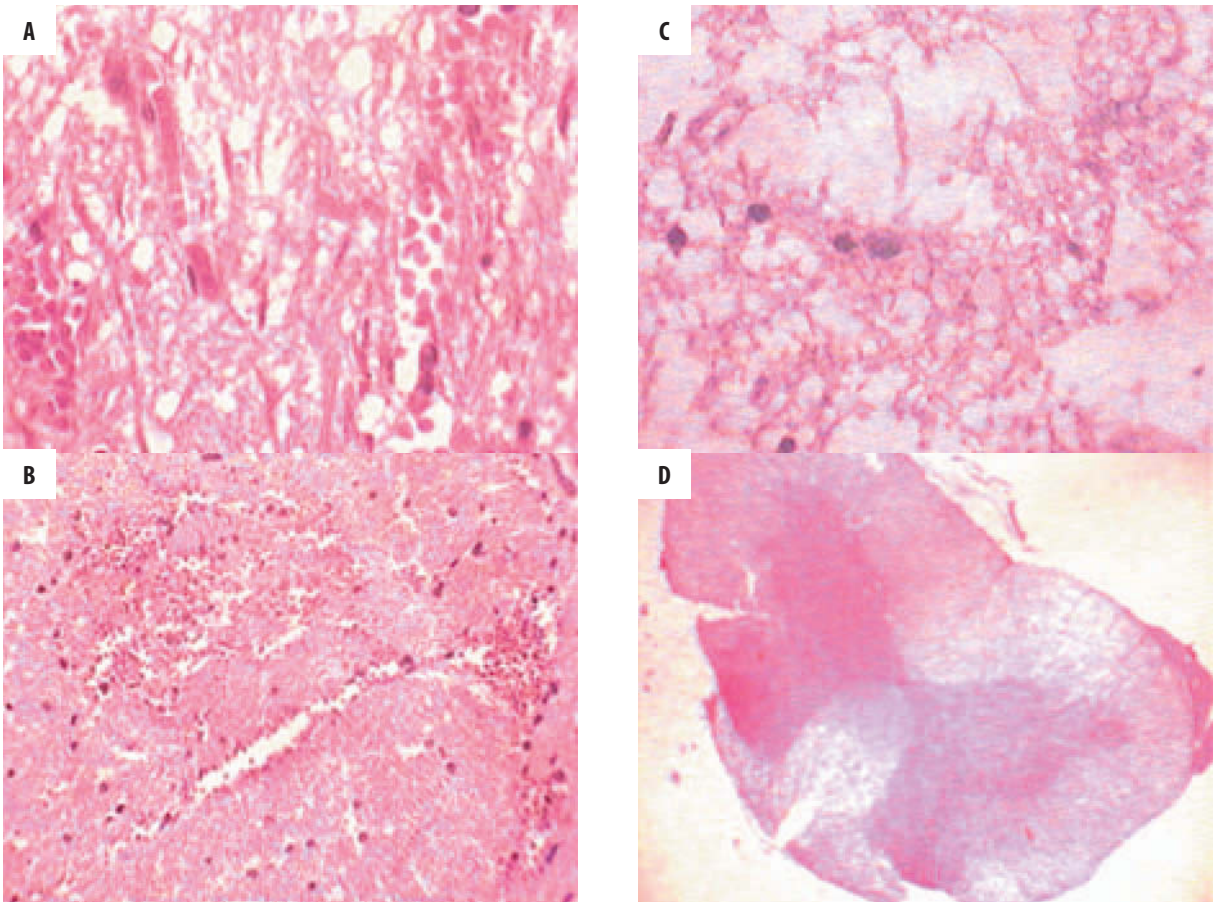


Figure 4. Some of the histopathological changes of white matter showing after H&E staining: (**A**) vascularization and/or increase in vascular density was prominent after 15Gy 26 weeks post-irradiation (×400), (**B**) congestion and gliosis after dose of 4Gy 13 weeks after irradiation (×100), (**C**) malacia after 25Gy 39 weeks after irradiation (×400), same manifestation of myelopathy was also seen in the group of 35Gy, and (**D**) spinal cord with deformed pattern and huge glioma after 35Gy of X-ray dose (×40).

Table 1. Microscopic and ultra-microscopic histopathological changes in white matter parenchyma and vascularity in rat cervical spinal cord observed (24 hours–39 weeks) after irradiation.

White matter parenchyma	Vascular changes	Ultra-microscopic changes in endothelial cells
– White matter necrosis, – Gliosis, – Myelin sheath irregularities – Demyelination of nerve fibre – Atrophy	– Vascular rupture and collapse, – Vascular density variation, – Thrombo-emboli formation, – Blood cell infiltration (haemorrhage)	– Cytoplasmic and basal cell membrane irregularities – Organelle morphological pattern – Vesiculation – Increase in number of lysosomes – Auto lysosome formation – Degeneration of mitochondria (Ballooned cristae) – Dilatation of endoplasmic reticulum

According to the present findings, low-dose irradiation that is insufficient for the induction of a white matter lesion may still induce a vascular lesion, possibly because of the difference in radiosensitivity between vascular endothelium and neuro-glial cells [6]. It was reported that vascular changes are not necessarily accompanied by morphological endothelial alterations. In fact, some of the changes may be degenerative changes related to injury from inflammation induced by radiation such as rupture of vessel walls, dilatation and infiltration of blood cells. Intra-vascular

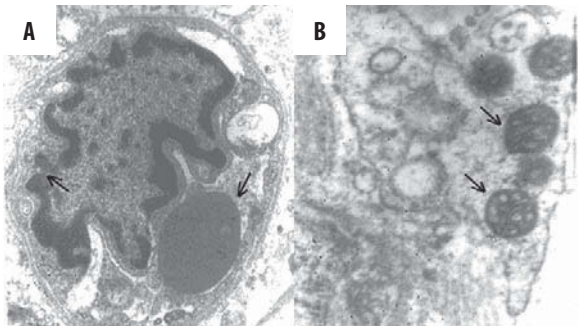


Figure 5. Transmission electron microscope images from rat spinal cord specimens: (**A**) Basal cell membrane detachment from the vessel wall (arrow) and deformed intracellular organelles (arrow head) are seen at 2Gy irradiation dose after 2 weeks (×12000), and (**B**) Mitochondrial destructive changes such as swelling and ballooned internal cristae (irreversible injury; arrows) with association of endoplasmic reticulum dilatation (arrows) at 30Gy (×30000) after 2 weeks are shown.

haemodynamic load can be deteriorated secondary to inflammation [17,18]. However, recently a dose-dependent loss of endothelial cells in rat cervical spinal cord at 24 hours after X-ray interaction was reported [19].

We found related injury from radiation-induced myelopathy such as blood vessels plugged with erythrocytes in and around the destroyed vessels, which may support the idea of changes in blood flow and endothelial surfaces. We also observed irregularities in the thickness of endothelial cell membrane (basement and cytoplasmic) with increased numbers of intracellular organelles, particularly vesicles early after irradiation, endoplasmic reticulum and degeneration of mitochondrial architecture (Figure 5).

Our chronological short- and long-term results further suggest that the response of the vascular tissues to radiation occurs prior to that of the nervous tissues of the spinal cord. Tissue damage is not the sum of cellular damage but an independent response of a highly structured tissue architecture and the most responsive substructure in the endothelial cell components. In addition, the response of the vascular system to radiation can be determined by the response of the endothelial cells, because the endothelial cells are introduced as the most radiosensitive among the cells constituting the vessel wall [6,18].

The pathological findings of radiation myelopathy are almost always confined to the damage of white matter and its vasculature [3,20]. Recently it has been suggested that radiation may alter the secretory profile of mediators, which can cause an inflammatory response, cellular proliferation, and cellular injury in the CNS [17,21]. Response of prostacyclin secretion to radiation should be considered in terms of its sensitivity to irradiation dose and kinetics. Finding out the existence of any relation between prostacyclin changes and induction of radiation toxicity needs to be based on knowledge of the physiologic effect of prostacyclin on the spinal cord [22].

Our previous report indicated that prostacyclin content decreased at 24 hours and 2 weeks post-irradiation followed by an increase at 13 weeks post irradiation [4]. In the present study we observed that the level of prostacyclin again decreased at 26 and 39 weeks after irradiation. Overall change in the profile of prostacyclin as a function of time has a bell shape. Change of prostacyclin as a function of time and dose is double-peaked (Figure 3A). This may be explained at the vascular level, since ionizing radiation stimulates the release of free arachidonic acid from membrane phospholipids through activation of phospholipases. This can cause an abrupt increase in the level of free arachidonic acid in the extra-cellular space and may induce a transient disruption of microvessel permeability measured in the spinal cord of irradiated rats. This may also confirm an earlier study of the effects of free radicals on inactivation of cyclo-oxygenase and prostacyclin synthase enzymes [21,22]. The steep down fluctuations in our study may be either secondary to exhaustion of the endogenous substrate or due to reduction in the activity of enzymes involved in prostaglandin synthesis. Late effects after low and high doses of irradiation showed a decrease in the concentration of prostacyclin compared to the age-matched control values. This type of unsteady dose dependency may be due to responses of different cell populations with different radiation responsiveness.

Conclusions

In summary, prostacyclin synthesis shows fluctuation with radiation dose and time. Very low radiation doses mainly affect endothelial cell functions, while neuroglial tissues are more resistant. High radiation doses affect mostly neuroglial tissues, inhibiting normal proliferation and differentiation. Low radiation doses of 2–6Gy increase production of prostacyclin at early times after irradiation. Single high doses of radiation show more complex fluctuations of prostacyclin in association with marked histopathological changes in neuroglial tissues of the spinal cord. Clearly it

can be postulated that induction of prostacyclin at certain times may assist the body to overcome late effects of radiation.

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